Suppressor of Cytokine Signaling 1/JAB and Suppressor of Cytokine Signaling 3/Cytokine-Inducible SH2 Containing Protein 3 Negatively Regulate the Signal Transducers and Activators of Transcription Signaling Pathway in Normal Human Epidermal Keratinocytes

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The suppressor of cytokine signaling/cytokine-inducible SH2 containing proteins are cytokine inducible and are negative regulators of the signal transducers and activators of the transcription signaling pathway. We investigated the mechanism regulating signal transducers and activators of transcription and the suppressor of cytokine signaling/cytokine-inducible SH2 containing protein family in keratinocytes, one of the major target cells for cytokines. Suppressor of cytokine signaling 1 mRNA was upregulated 3 h post-interferon γ, and a 8.1-fold increase in the suppressor of cytokine signaling 1 mRNA occurred 48 h post-interferon γ. The suppressor of cytokine signaling 3 mRNA was also upregulated from 1 h post-interferon γ, and a 6.7-fold increase in the suppressor of cytokine signaling 3/cytokine-inducible SH2 containing protein 3 mRNA occurred between 6 and 12 h post-interferon γ. Interleukin-6 exposure for 1 h enhanced the expression of the suppressor of cytokine signaling 3/cytokine-inducible SH2 containing protein 3 mRNA, but the suppressor of cytokine signaling 1/JAB mRNA was not induced by interleukin-6. Interleukin-4 upregulated the suppressor of cytokine signaling 1/JAB and cytokine-inducible SH2 containing protein 1 mRNA, with 3.4-fold and 5.1-fold increases in mRNA observed at 1 h post-interleukin-4, respectively. In contrast, epidermal growth factor, which phosphorylates signal transducers and activators of transcription 3, did not influence the level of the suppressor of cytokine signaling/cytokine-inducible SH2 containing protein family mRNA expression. Transfection of an adenovirus vector expressing the suppressor of cytokine signaling 1/JAB completely inhibited interferon γ-dependent signal transducers and activators of transcription 1 phosphorylation and interleukin-4-dependent signal transducers and activators of transcription 6 phosphorylation. Transfection of adenovirus vector expressing the suppressor of cytokine signaling 1/JAB did not inhibit interleukin-6-dependent signal transducers and activators of transcription 3 phosphorylation—several reports show that the suppressor of cytokine signaling 1/JAB is a potent inhibitor of signal transducers and activators of transcription 3 signaling in the myeloid leukemia M1 cell. Transfection of the adenovirus vector expressing suppressor of cytokine signaling 3/cytokine-inducible SH2 containing protein 3 completely inhibited interleukin-6-dependent signal transducers and activators of transcription 3 phosphorylation and partially inhibited interferon γ-dependent signal transducers and activators of transcription 1 phosphorylation. Transfection of the adenovirus vector expressing suppressor of cytokine signaling 3/cytokine-inducible SH2 containing protein 3, however, did not inhibit interleukin-4-dependent signal transducers and activators of transcription 6 phosphorylation. Transfection of the adenovirus vector expressing cytokine-inducible SH2 containing protein 1 had no effect on signal transducers and activators of transcription 1, 3, and 6 signaling in normal keratinocytes. Therefore, the relationship between signal transducers and activators of transcription and suppressor of cytokine signaling is unique in the keratinocytes, and the suppressor of cytokine signaling regulates cytokine signals in these cells. ***Key words: Keratinocyte/SOCS/CIS/STAT/interferon gamma/Interleukin-4/Interleukin-6. J Invest Dermatol 120:571–580, 2003***

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Abbreviations: SOCS, suppressor of cytokine signaling; CIS, cytokine-inducible SH2 containing protein; STAT, signal transducers and activators of transcription; JAB, JAK-binding protein; JAK, Janus-activated kinase.

The skin is composed of the epidermis and the dermis, and keratinocytes are the major cells of the epidermis. Many different cytokines influence the dynamics of keratinocyte function, including interferon (IFN)-γ, interleukin (IL)-4, and IL-6 (Furue, 1994; Ricci, 1994; Fransson *et al*, 1996; Huang *et al*, 2001). IFN-γ is

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secreted from T helper (Th)1 lymphocytes, and is a possible psoriasis pathogen (Schlaak *et al*, 1994). IFN-γ induces the expression of molecules that recruit and activate T cells and other leukocytes in the skin (Barker *et al*, 1990). Th2-derived IL-4 is abundantly expressed in atopic dermatitis (Del Prete, 1992; Furue, 1994; Ricci, 1994). The balance between Th1 and Th2 cells affects both keratinocyte growth and the expression of chemoattractants by keratinocytes (Fransson *et al*, 1996; Vestergaard *et al*, 2000). Many aspects of the biologic functions of these cytokines have been investigated, but their role in regulating intracellular signal transduction in human keratinocytes is not fully understood. Recent studies indicate that signal transducers and activators of transcription (STAT), and their negative regulators, the suppressor of cytokine signaling (SOCS)/cytokine-inducible SH2 containing protein (CIS) family of proteins, play a central role in this regulatory mechanism in various different cell types.

STAT are crucial molecules for the cytokine-signaling pathway. STAT transmit cytokine-derived signals to the nucleus of the cell (Bromberg and Darnell, 2000). Seven isotypes of STAT molecules have been identified: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. STAT1 and STAT2 transduce signals from IFN-α, β, and γ. STAT3 transduces gp130 receptor signaling, which is activated by IL-6 and IL-10. Epidermal growth factor (EGF) and platelet-derived growth factor also phosphorylate STAT3. STAT4 is an important molecule in the IL-12 signaling pathway, and STAT5a and STAT5b are stimulated by IL-2 and erythropoietin. STAT6 is important for IL-4 and IL-13 signaling. After a cytokine binds to its receptor, Janus-activated kinase (JAK) is phosphorylated (Imada and Leonard, 2000; Kotenko and Pestka, 2000; Gadina *et al*, 2001). Then, JAK, in turn, phosphorylates the cytokine receptor. STAT binds to the phosphorylated receptor, and STAT is subsequently phosphorylated by JAK. The phosphorylated STAT molecule then forms a homodimer or heterodimer, and translocates into the nucleus, where it initiates the transcription of a candidate gene.

Recently, the SOCS/CIS family and the protein inhibitor of activated STAT were isolated as negative regulators or modulators of the cytokine/STAT signaling pathway(Duhe *et al*, 2001). The protein inhibitor of activated STAT is found constitutively in the cytoplasm and regulates the translocation of phosphorylated STAT to the nucleus by binding to phosphorylated STAT (Liu *et al*, 1998). The SOCS/CIS family is inducible *de novo* by cytokine stimulation. Synthesized SOCS/CIS protein binds to tyrosine phosphorylated sites of the cytokine receptor or JAK through an SH2 domain, and consequently inhibits phosphorylation of tyrosine kinase. Yoshimura *et al* (1995) first isolated CIS as an erythropoietin signal inhibitor that binds to the tyrosine-phosphorylated site of the erythropoietin receptor and inhibits the downstream signal of STAT5 activation. New members of the SOCS/CIS family have been identified independently (Endo *et al*, 1997; Naka *et al*, 1997; Starr *et al*, 1997). SOCS1/JAB mainly binds to JAK2 (Yasukawa *et al*, 1999) and works as a crucial molecule regulating IFN-γ/STAT1 (Alexander *et al*, 1999) and IL-6/STAT3 signaling in some cell types (Naka *et al*, 1997). SOCS2/CIS2 is predicted to be a critical regulator of insulin-like growth factor and the insulin-like growth factor receptor signal (Metcalf *et al*, 2000) based on evidence from the SOCS2/CIS2 knockout mouse, which showed gigantism as an abnormal phenotype. SOCS3/CIS3 is reported to regulate the IL-6/STAT3 and IFN-γ/STAT1 signaling pathways. The functions of the other members of the SOCS/CIS family, SOCS4–SOCS7, remain unclear.

The relationship between cytokines and the SOCS/CIS family and the inhibitory profile of the SOCS/CIS family differ among cell types. The inhibitory and negative regulatory mechanisms of cytokine signals in epidermal keratinocytes have not been fully assessed, although cytokines such as IFN-γ, IL-4, and IL-6 are implicated in a variety of physiologic and pathologic conditions of the skin.

We studied SOCS/CIS function in normal human epidermal keratinocytes using an adenovirus expression system. The results suggest that SOCS is one of the major negative regulators of the cytokine/STAT signaling pathway in human keratinocytes.

MATERIALS AND METHODS

Cell culture Normal human skin was obtained from plastic surgery patients, cut into 3–5 mm strips, and incubated with 250 U per ml of dispase in Dulbecco's modified Eagle's medium overnight at 4°C. The epidermis was separated from the dermis with forceps, and the epidermal sheets were rinsed with phosphate-buffered saline, incubated in a 0.25% trypsin solution for 10 min at 37°C, and then teased with forceps. Epidermal cells were rinsed and collected by centrifugation. Normal human keratinocytes were cultured in MCDB153 medium supplemented with insulin (5 μg per ml), hydrocortisone (5×10^{-7} M), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), bovine hypothalamic extract (50 μg per ml), and Ca^{2+} (0.03 mM). This supplement has been described elsewhere (Sayama *et al*, 1999). Cells were counted using a Coulter™ Z1 Cell Counter (Coulter, Tokyo, Japan). All procedures involving human subjects received prior approval from the ethical committee of Ehime University School of Medicine, and all subjects provided informed written consent.

Reagents Recombinant IFN-γ and EGF were generous gifts from Otsuka Pharmaceutical Co. Ltd. (Tokyo, Japan). Recombinant IL-6 and IL-4 were obtained from PeproTech EC Ltd. (London, U.K.).

Anti-phosphoSTAT1 (α-pSTAT1), anti-phosphoSTAT3 (α-pSTAT3), and anti-phosphoSTAT6 (α-pSTAT6) antibodies were purchased from New England Biolabs (Beverly, MA). Anti-STAT1 (α-STAT1) and anti-STAT3 (α-STAT3) antibodies were purchased from Promega (Tokyo, Japan). Anti-STAT6 (α-STAT6) antibody was purchased from Transduction Laboratories (Lexington, KY).

Oligonucleotide probe preparation Polymerase chain reaction amplified human cDNA were inserted into the *Eco*RI and *Hin*dIII sites of the pPMG vector (PharMingen, San Diego). Inserted cDNA corresponded to oligonucleotides 550–769 of STAT1 (accession no. M97935), 345–586 of STAT2 (accession no. M97934), 1846–2109 of STAT3 (accession no. L29277), 1824–2109 of STAT4 (accession no. L78440), 1773–2085 of STAT5a (accession no. U43185), 2115–2448 of STAT5b (accession no. U47686), 1500–1854 of STAT6 (accession no. U16031), 395–554 of CIS1 (accession no. AF132297), 351–525 of SOCS1/JAB (accession no. AB005043), 83–273 of SOCS2/CIS2 (accession no. AB004903), and 556–764 of SOCS3/CIS3 (accession no. AB004904). These inserted cDNA were confirmed by nucleotide sequencing. The pPMG vector, including GAPDH cDNA (PharMingen), was used as the internal standard.

Ribonuclease protection assay Keratinocytes derived from three independent donors were used for ribonuclease protection assays. Total cellular RNA was isolated from cultured human keratinocytes using Isogen® (Nippon Gene, Toyama, Japan).

Single-stranded anti-sense riboprobes were prepared by *in vitro* transcription of human cDNA fragments, using a RiboQuant® In Vitro Transcription kit (PharMingen) in the presence of [α-^{32}P]uridine triphosphate. Samples of total RNA (10 μg each) were hybridized with ^{32}P-labeled riboprobe and digested with RNase using the RiboQuant® ribonuclease protection assay kit according to the manufacturer's instructions. Hybridization products were separated on a 5% polyacrylamide/8 M urea gel. The gel was absorbed on filter paper, dried, and then exposed to Kodak BiomaxMS® film (Kodak, Japan) at −70°C. Band density was analyzed using the Diversity Database™ (PDI Inc., New York). Relative values of SOCS/CIS mRNA were estimated using GAPDH as an internal reference, and normalized against the respective relative value at 0 h poststimulation. Reproducibility was confirmed by performing three independent experiments using keratinocytes derived from three independent donors. Typical results were represented as photo images. The mean and standard deviation of relative values obtained from three independent experiments using keratinocytes derived from three independent donors were plotted on graphs.

Adenovirus vector construction and infection The cosmid cassettes pAxCAw (Miyake *et al*, 1996) and pAxCALNLw (Kanegae *et al*, 1996), nuclear localizing signal-tagged Cre recombinase-expressing adenovirus (AxCANCre), control adenovirus Ax1w, and the parent virus Ad5-dLX (Miyake *et al*, 1996) were all kind gifts from Dr Izumu Saito (Tokyo University, Japan). The full-length coding regions of STAT1W, STAT1F, STAT3W, STAT3F, and SOCS3/CIS3 cDNA were subcloned into the adenovirus cosmid cassette pAxCAw. STAT1W, and STAT3W code

normal STAT1 and STAT3 sequences, respectively. STAT1F and STAT3F code dominant negative mutants that have their tyrosine phosphorylation sites changed to phenylalanine. The full-length coding regions of CIS1 and SOCS1/JAB cDNA were subcloned into pAxCALNLw. Adenovirus containing the CA promoter and STAT1W, STAT1F, STAT3W, STAT3F, or CIS3 (AxCAStat1w, AxCAStat1F, AxCAStat3w, AxCAStat3F, and AxCACIS3, respectively), and adenovirus containing the CALNL unit and CIS1 or JAB (AxCALNLCIS1 and AxCALNLJAB, respectively) were generated using the COS-TPC method (Miyake *et al*, 1996). The cosmid DNA was mixed with the *Eco*T22I-digested DNA-terminal Ad5-dLX protein complex, and used to cotransfect 293 cells. Recombinant viruses were generated through homologous recombination in 293 cells. Virus stocks were prepared using a standard procedure (Miyake *et al*, 1996). Concentrated, purified virus stocks were prepared by a CsCl gradient, and the virus titer was checked by plaque formation assay.

Normal human keratinocytes were infected with adenovirus vectors at a multiplicity of infection of 5. AxCALNLJAB and AxCALNLCIS1 were transfected with AxCANCre, because they can generate SOCS1/JAB and CIS1 mRNA under the influence of Cre recombinase. The expression of these genes was confirmed with reverse transcription–polymerase chain reaction using the specific probes described above.

Western blot analysis Cells were harvested by scraping with extraction buffer, containing 150 mM NaCl, 1% nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris–HCl pH 7.4, and protease inhibitors. Equal amounts of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes. The first antibodies were incubated overnight at 4°C. Fluorescein-labeled goat anti-mouse or rabbit IgG (Amersham Life Science, Inc., Arlington Heights, IL) were used as the second antibody. The signal was amplified with an anti-fluorescein antibody followed by a fluorescent substrate, AttoPhos (Amersham Life Science, Inc.). The membrane was then scanned using FluoroImager (Molecular Dynamics Inc., Sunnyvale, CA). The intensity of each band was quantified with ImageQuant™ (Molecular Dynamics Inc.), with reference to the control signal as one unit. Reproducibility was confirmed by performing three independent experiments using keratinocytes derived from three independent donors.

RESULTS

Upregulation of SOCS1/JAB and SOCS3/CIS3 mRNA by IFN-γ We first examined the effect of IFN-γ on STAT and SOCS/CIS expressions in primary keratinocytes. IFN-γ is a potent anti-proliferating agent of primary keratinocytes (Nickoloff *et al*, 1984), and phosphorylates STAT1 and STAT2 (Bromberg and Darnell, 2000). Following the application of IFN-γ, cells were harvested at the indicated time and total RNA was analyzed by a ribonuclease protection assay (**Fig 1*a***). In quiescent keratinocytes, STAT1, STAT2, STAT3, STAT5b, and STAT6 were dominantly expressed. IFN-γ enhanced STAT1 and STAT2 mRNA expression in a time-dependent manner. IFN-γ had no effect on the expression of STAT3, STAT5b, or STAT6 mRNA. Quiescent keratinocytes did not express STAT4 or STAT5a mRNA, but STAT5a mRNA was induced 12–48 h post-IFN-γ, and STAT4 was induced 24–48 h post-IFN-γ. Along with the induction of STAT1 and STAT2 mRNA, SOCS1/JAB and SOCS3/CIS3 mRNA were expressed in a time-dependent manner. SOCS3/CIS3 mRNA was rapidly upregulated 1 h post-IFN-γ, and was increased 6.7-fold at 6–12 h post-IFN-γ (**Fig 1*b***). SOCS1/JAB mRNA was detectable 3 h post-IFN-γ and increased 8.1-fold after 48 h of IFN-γ exposure (**Fig 1*c***). SOCS2/CIS2 mRNA was downregulated by IFN-γ (**Fig 1*d***), and CIS1 levels were induced 3–4-fold 1–48 h post-IFN-γ (**Fig 1*e***).

Upregulation of SOCS3/CIS3 mRNA but not SOCS1/JAB mRNA by IL-6 IL-6 phosphorylates STAT3 in keratinocytes (Sano *et al*, 1999; Hirano *et al*, 2000). In contrast to IFN-γ, IL-6 did not alter the expression of STAT family mRNA in keratinocytes (**Fig 2*a***); however, IL-6 strongly enhanced the expression of SOCS3/CIS3 mRNA, with a 8.7-fold increase at 1 h post-IL-6 (**Fig 2*b***). Furthermore, SOCS3/CIS3 expression was slightly decreased at 3 h, and then increased 2–3-fold from 6 to 48 h. SOCS1/JAB mRNA is not induced by IL-6 stimulation in keratinocytes (**Fig 2*c***). The expression of CIS1 and SOCS2/CIS2 mRNA were not affected by IL-6 (**Fig 2*d,e***, respectively).

Upregulation of SOCS1/JAB and CIS1 mRNA by IL-4 IL-4 is produced by Th2 cells in atopic skin conditions (Ricci, 1994), and the IL-4 signal is transduced by STAT6 (Takeda *et al*, 1996, 1997). IL-4 stimulation did not alter the expression of STAT family mRNA in keratinocytes, as did IL-6 stimulation (**Fig 3*a***). A 3.5-fold increase in SOCS1/JAB mRNA was induced 1 h post-IL-4 in keratinocytes (**Fig 3*c***). IL-4-dependent SOCS1/JAB induction seemed to be biphasic, with peaks at 1–3 h and 12–36 h. A 4.8-fold increase in CIS1 mRNA was also induced 1 h post-IL-4 (**Fig 3*e***). IL-4-dependent CIS1 induction was also biphasic, with peaks at 1–3 h and 24–48 h. IL-4 had no effect on SOCS2/CIS2 and SOCS3/CIS3 (**Fig 3*b,d***, respectively).

Effect of EGF on SOCS/CIS family mRNA EGF is a potent mitogen for keratinocytes, and it was reported that EGF phosphorylates the STAT3 and MAP kinase family (Olayioye *et al*, 1999). EGF induced a transient increase in STAT1, STAT2, and STAT3 mRNA 1 h after keratinocyte stimulation (**Fig 4*a***). After a 1 h stimulation, EGF also induced a slight increase in SOCS1/JAB mRNA (**Fig 4*c***), and downregulated SOCS3/CIS3 mRNA. In contrast, IFN-γ and IL-6 upregulated SOCS3/CIS3 mRNA (**Fig 4*b***). EGF did not alter the expression of CIS1 and SOCS2/CIS2 mRNA (**Fig 4*d* and 4*e***, respectively).

Effect of SOCS1/JAB on IFN-γ-induced STAT1 phosphorylation We demonstrated that cytokines such as IFN-γ, IL-6, and IL-4 induce their negative regulators, CIS1, SOCS1/JAB, and SOCS3/CIS3, in keratinocytes; however, it has not been proven whether this SOCS family of proteins is able to inhibit the cytokine-signaling pathway. To study the functional role of SOCS/CIS proteins that are expressed in keratinocytes, we used an adenovirus vector expressing normal CIS1 (AxCALNLCIS1), normal SOCS1/JAB (AxCALNLJAB), and normal SOCS3/CIS3 (AxCACIS3).

STAT1 is phosphorylated by IFN-γ. We examined the effect of CIS1, SOCS1/JAB, and SOCS3/CIS3 on IFN-γ-induced STAT1 phosphorylation levels in keratinocytes. The adenovirus vectors were added to medium containing keratinocytes and incubated for 24 h to induce SOCS/CIS protein production. Then, keratinocytes were stimulated with IFN-γ. The proteins extracted from keratinocytes (treated or untreated with IFN-γ) were analyzed by western blot analysis.

AxCALNLJAB completely inhibited IFN-γ-induced STAT1 phosphorylation in keratinocytes, whereas AxCACIS3 weakly reduced the STAT1 phosphorylation level (**Fig 5*a***). AxCIS1 had no effect on IFN-γ-induced STAT1 phosphorylation. Time-course studies of AxCALNLJAB- and AxCACIS3-transfected keratinocytes confirmed that IFN-γ-induced STAT1 phosphorylation was inhibited by SOCS1/JAB (**Fig 5*b,c***). These data indicate strong inhibition of IFN-γ-induced STAT1 phosphorylation by SOCS1/JAB, and weak inhibition by SOCS3/CIS3.

Inhibition of IL-6-induced STAT3 phosphorylation by SOCS3/CIS3 We examined the effect of CIS1, SOCS1/JAB, and SOCS3/CIS3 on IL-6-induced STAT3 phosphorylation using an adenovirus vector system as described above. AxCACIS3 completely inhibited IL-6-induced STAT3 phosphorylation in keratinocytes (**Fig 6*a***). This observation is consistent with previous reports involving myeloid leukemia M1 cells (Endo *et al*, 1997; Naka *et al*, 1997; Starr *et al*, 1997). Interestingly, AxCALNLJAB did not affect IL-6-dependent STAT3 phosphorylation in keratinocytes. This result does not agree with M1 cell observations; in these cells SOCS1/JAB works as a potent inhibitor of the IL-6-induced STAT3 signaling pathway (Endo *et al*, 1997; Naka *et al*, 1997; Starr *et al*, 1997). Time-course studies of

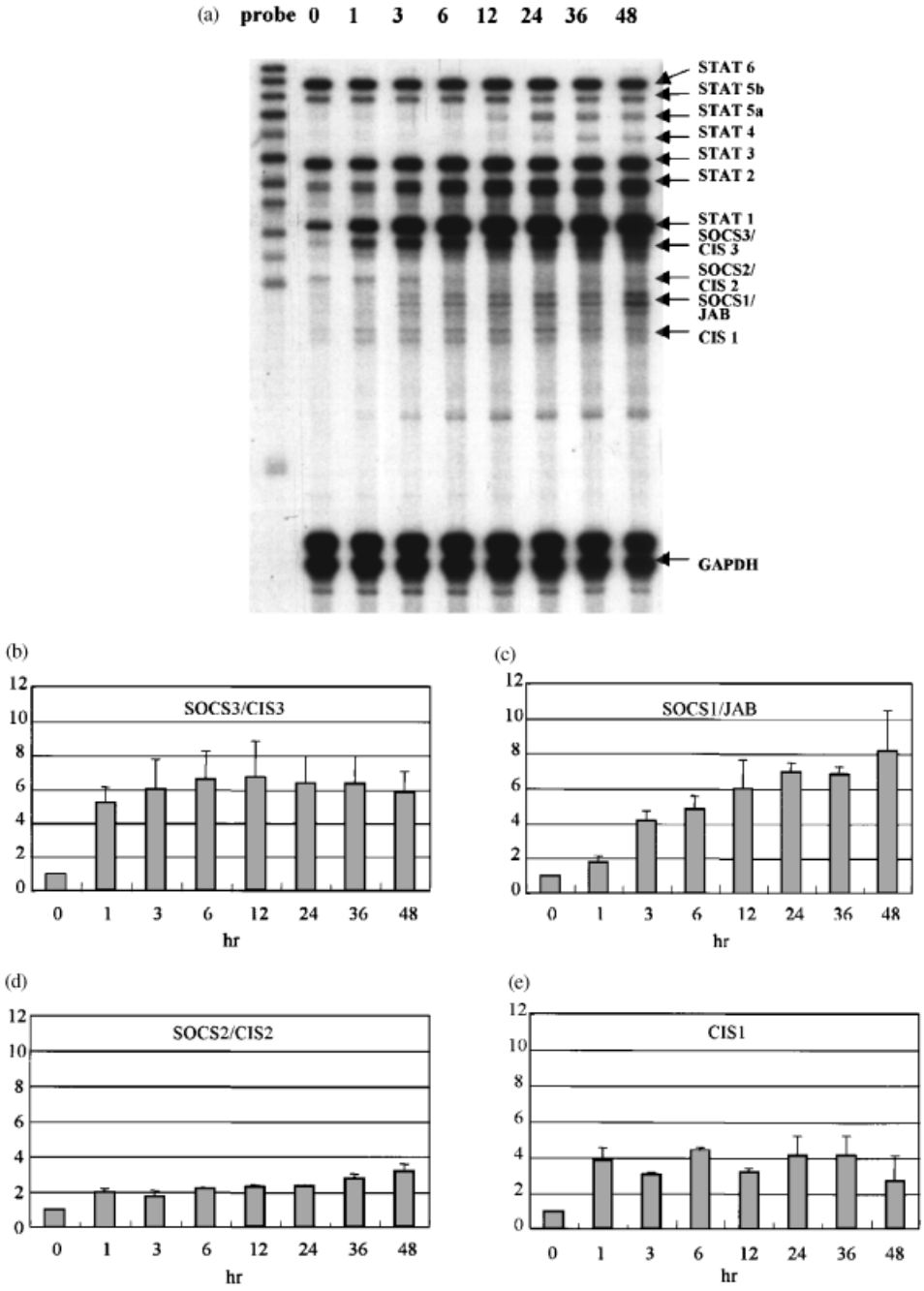

Figure 1. Induction of SOCS/CIS mRNA by IFN-γ in keratinocytes. IFN-γ (10 IU per ml) was added to keratinocytes in culture medium, and total RNA was extracted at 0, 1, 3, 6, 12, 24, 36, and 48 h after stimulation. Total RNA was hybridized with RNA probes, and separated on a polyacrylamide gel. *Arrows* on the right side of the panel indicate the predicted mRNA bands for STAT and SOCS (*a*). The density of each band was analyzed using the Diversity Database™ (PDI Inc., New York). Relative values for SOCS3/CIS3 (*b*), SOCS1/JAB (*c*), SOCS2/CIS2 (*d*), and CIS1 mRNA (*e*) were estimated using GAPDH as an internal reference, and normalized against the respective relative value at 0 h poststimulation. Each bar represents the mean of relative values obtained from three independent experiments using keratinocytes derived from three independent donors. The error bars indicate standard deviations.

AxCALNLJAB- and AxCACIS3-transfected keratinocytes confirmed that IL-6-induced STAT3 phosphorylation was not inhibited by SOCS1/JAB (**Fig 6*b,c***). CIS1 had no effect on IL-6-induced STAT3 phosphorylation. As IL-6 induced SOCS3/CIS3 mRNA expression only in keratinocytes (as shown in **Fig 2*a***), SOCS3/CIS3 may be a major negative regulator in the keratinocyte IL-6-induced STAT3 signaling pathway.

Inhibition of IL-4-induced STAT6 phosphorylation by SOCS1/JAB STAT6 is an essential signal transducer of IL-4 stimulation. The inhibitory effect of SOCS/CIS proteins on IL-4-induced STAT6 phosphorylation was studied. IL-4 enhanced the expression of SOCS1/JAB and CIS1 mRNA (**Fig 3*a***). AxCALNLJAB inhibited IL-4-dependent STAT6 phosphorylation (**Fig 7*a,b***); however, CIS1 did not affect IL-4-induced STAT6 phosphorylation, although CIS1 is upregulated by IL-4 stimulation (**Fig 7*a,c***). Although a previous study shows SOCS3/CSI3 inhibit IL-4-dependent STAT6 activation (Haque *et al*, 2000), SOCS3/CIS3 had no effect on IL-4-induced STAT6 phosphorylation in human keratinocytes. Hence, the SOCS1/JAB protein is a major negative regulator of IL-4-induced STAT6 activation.

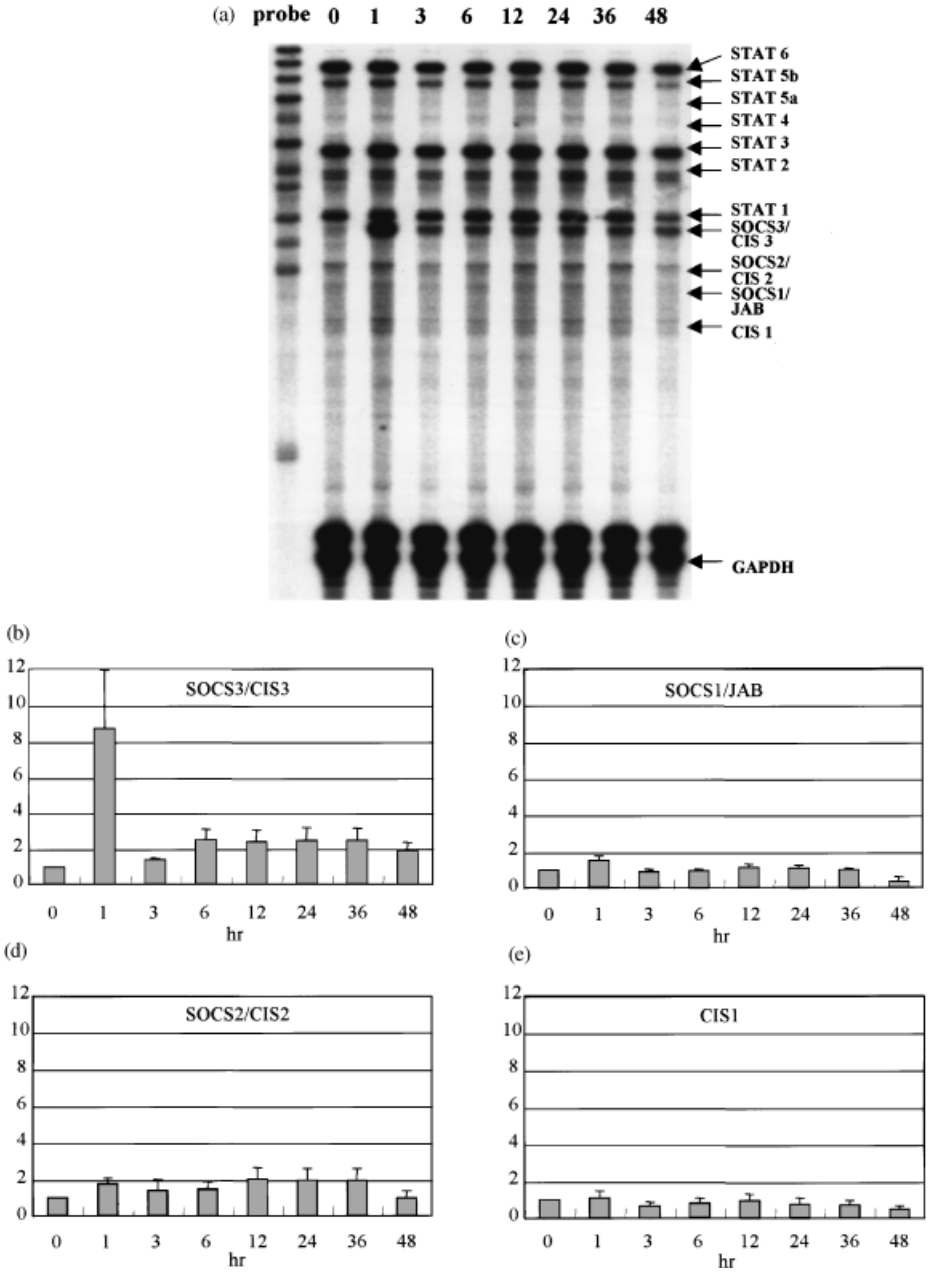

Figure 2. Induction of SOCS/CIS mRNA by IL-6 in keratinocytes. IL-6 (10 ng per ml) was added to keratinocytes in culture medium, and total RNA was extracted at 0, 1, 3, 6, 12, 24, 36, and 48 h after stimulation. A gel image of a ribonuclease protection assay is shown (*a*), and relative values for SOCS3/CIS3 (*b*), SOCS1/JAB (*c*), SOCS2/CIS2 (*d*), and CIS1 mRNA (*e*) were estimated as described in the legend of **Fig 1**.

Keratinocyte-specific negative feedback profile of SOCS1/JAB SOCS1/JAB is a potent negative regulator of IL-6-induced STAT3 phosphorylation in leukemia cells, but not in keratinocytes. This keratinocyte-specific characteristic of SOCS1/JAB was analyzed. To confirm whether SOCS1/JAB inhibited the IL-6-induced signaling pathway, we examined the effect of AxCALNLJAB on the SOCS3/CIS3 mRNA expression induced by IFN-γ and IL-6. AxCALNLJAB inhibited IFN-γ-dependent SOCS3/CIS3 mRNA induction, as did AxCASTAT1F, a dominant negative STAT1 (**Fig 8*a***). On the other hand, AxCASTAT3F, a dominant negative STAT3, inhibited IL-6-dependent SOCS3/CIS3 mRNA induction, whereas AxCALNLJAB did not (**Fig 8*c***). Time-course studies of AxCALNLJAB-transfected keratinocytes confirmed that IFN-γ-dependent SOCS3/CIS3 induction was inhibited by SOCS1/JAB, but IL-6-dependent induction was not (**Fig 8*b,d***). These data verify that SOCS1/JAB does not inhibit IL-6-induced STAT3 activation in keratinocytes.

DISCUSSION

Binding of IFN-γ to its receptor activates STAT1. Phosphorylated STAT1 forms a homodimer and transduces the IFN-γ signal in keratinocytes, inducing the expression of the intercellular adhesion molecule-1, and enhancing the allergic reaction (Jiang *et al*, 1994; Naik *et al*, 1997). STAT2 forms a heterodimer with STAT1 that associates with p48 to transduce the IFN-α/β signal (Schindler and Darnell, 1995). Although the specific function of STAT2 in keratinocyte biology is not well understood, keratinocytes produce IFN-β (Fujisawa *et al*, 1997), and STAT2 may transduce the IFN-α/β signal in keratinocytes. Whereas keratinocytes derived from STAT3-deficient mice have impaired migration (Sano *et al*, 1999), specific functions for STAT4, STAT5a, and STAT5b in keratinocytes have not been reported. STAT6 may transduce IL-4 and IL-13 signals, and modulate the expression of CD60w, a T cell costimulatory factor (Huang *et al*, 2001). Thus, in keratinocytes, STAT signal modulation may influence the response to

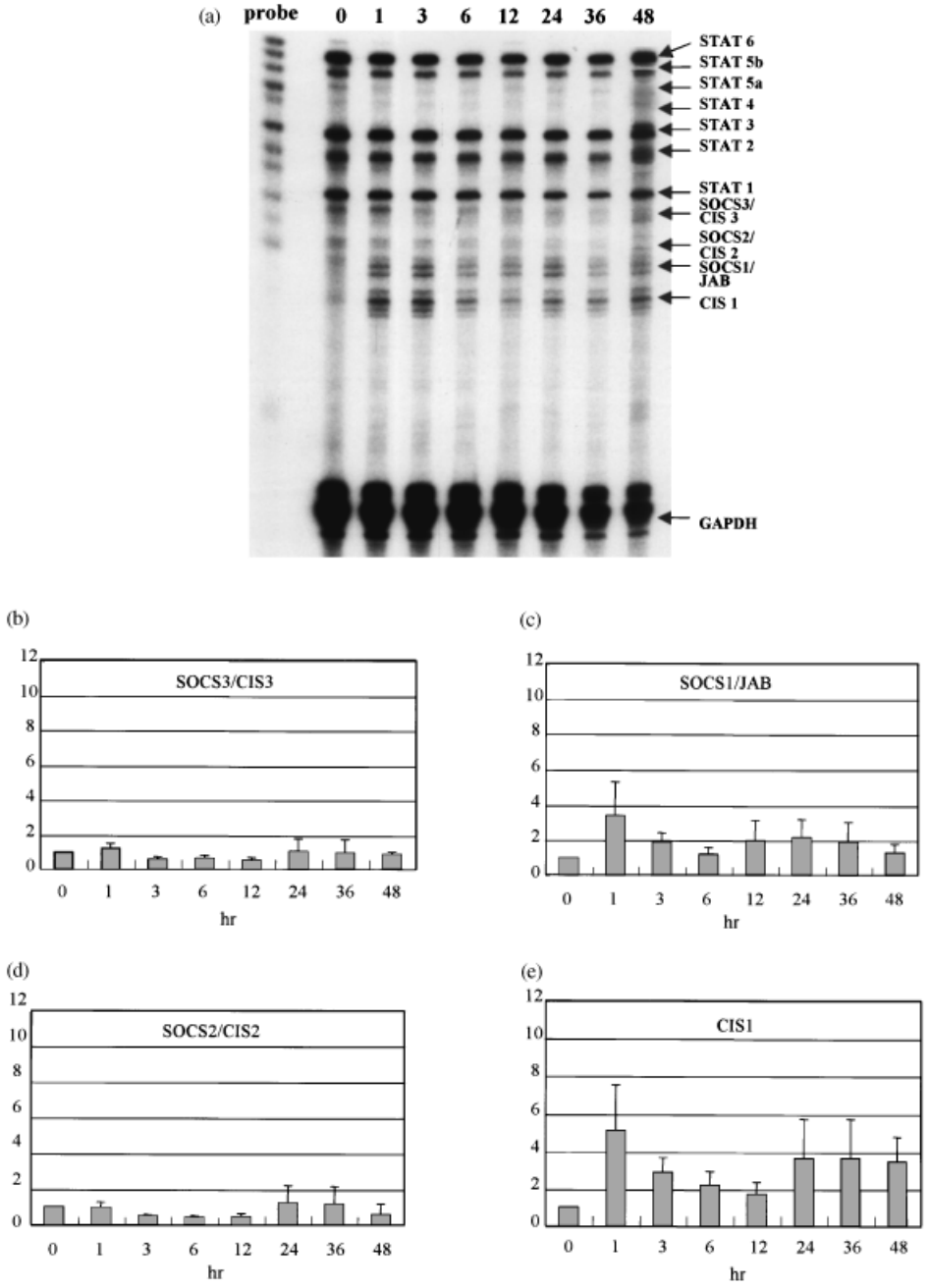

Figure 3. Induction of SOCS/CIS mRNA by IL-4 in keratinocytes. IL-4 (10 ng per ml) was added to keratinocytes in culture medium, and total RNA was extracted at 0, 1, 3, 6, 12, 24, 36, and 48 h after stimulation. A gel image of a ribonuclease protection assay is shown (*a*), and relative values for SOCS3/CIS3 (*b*), SOCS1/JAB (*c*), SOCS2/CIS2 (*d*), and CIS1 mRNA (*e*) were estimated as described in the legend of **Fig 1**.

inflammatory signals and mobility. The SOCS/CIS family is induced by cytokine stimulation and acts as a negative regulator of the cytokine signal, which leads to the suppression of STAT activation. Therefore, SOCS/CIS family induction may be one of the key regulatory mechanisms in the skin inflammatory reaction.

Strong IFN-γ induction of STAT1 and STAT2 may indicate the importance of these molecules in the IFN-γ signaling pathway and IFN-γ effect on keratinocytes. Along with the induction of STAT1 and STAT2 mRNA, SOCS1/JAB and SOCS3/CIS3 mRNA were expressed in human keratinocytes (**Fig 1*a***). After IFN-γ stimulation, SOCS3/CIS3 mRNA expression preceded that of SOCS1/JAB; SOCS3/CIS3 mRNA was detected 1 h after exposure to IFN-γ, and SOCS1/JAB was detected after 3 h (**Fig 1*a***). Thus, IFN-γ-induced SOCS3/CIS3 mRNA expression preceded SOCS1/JAB mRNA induction. IFN-γ-induced STAT1 phosphorylation was strongly inhibited by SOCS1/JAB and weakly inhibited by SOCS3/CIS3 (**Fig 5*a***). These results indicate that the SOCS3/CIS3 induced earlier may have partially inhibited IFN-γ-dependent STAT1 phosphorylation, and SOCS1/JAB induction following SOCS3/CIS3 had a potent inhibitory effect on STAT1 activation, and also inhibited the anti-proliferative effect of IFN-γ. These phenomena represent the dual negative regulatory system of the SOCS family following IFN-γ stimulation in keratinocytes: SOCS3/CIS3 first inhibits or modulates weak IFN-γ stimulation. If keratinocytes are exposed to IFN-γ for a long period, SOCS1/JAB is induced and blocks the IFN-γ signaling pathway intensively. Thus, IFN-γ stimulation is counteracted by SOCS3/CIS3 at an early phase and by SOCS1/JAB at a late phase. Therefore, sequential negative regulation by SOCS3/CIS3 and SOCS1/JAB may play a central part in the keratinocyte IFN-γ/STAT1 signaling pathway.

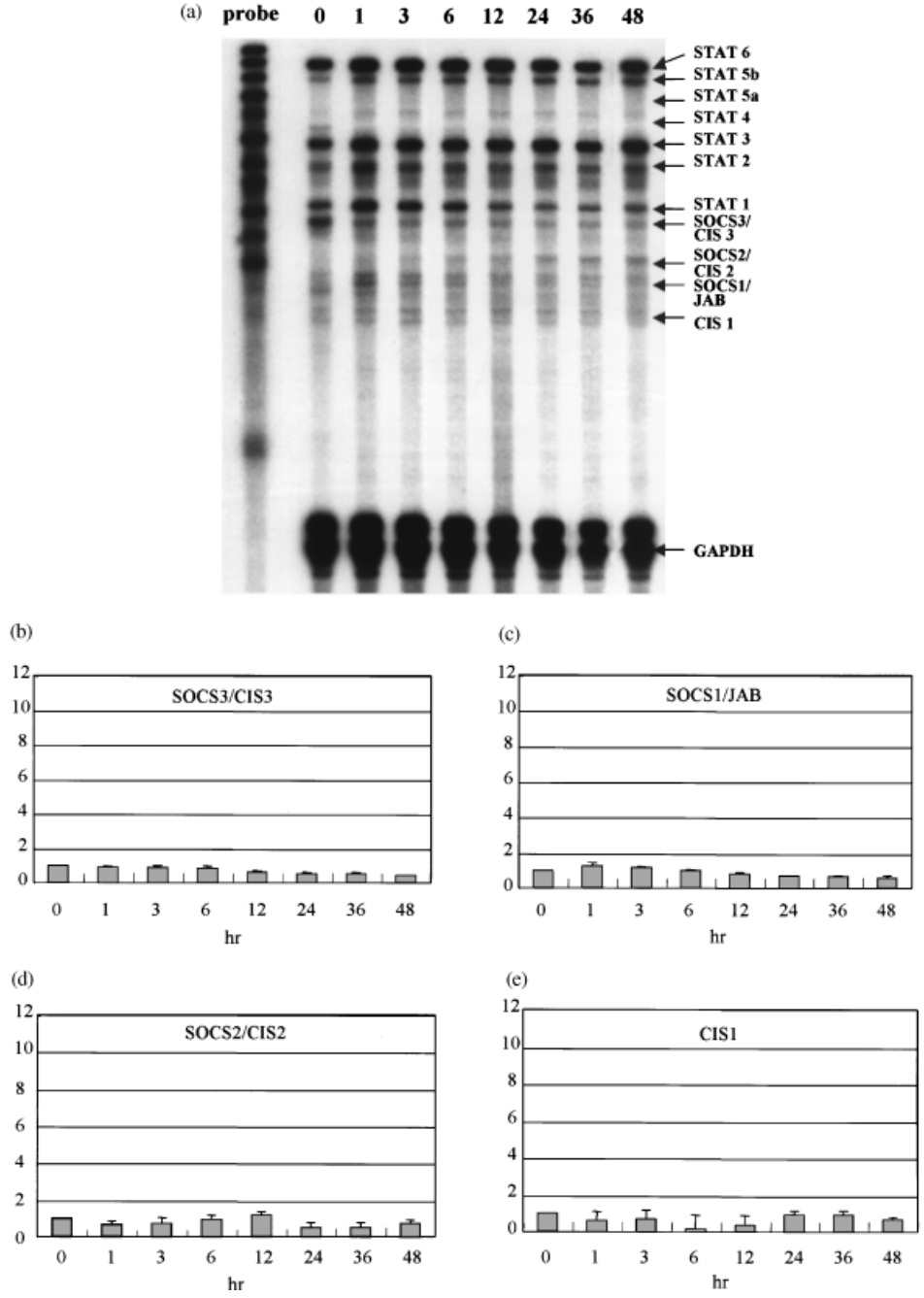

Figure 4. Induction of SOCS/CIS mRNA by EGF in keratinocytes. EGF (10 ng per ml) was added to keratinocytes in culture medium, and total RNA was extracted at 0, 1, 3, 6, 12, 24, 36, and 48 h after stimulation. A gel image of a ribonuclease protection assay is shown (*a*), and relative values for SOCS3/CIS3 (*b*), SOCS1/JAB (*c*), SOCS2/CIS2 (*d*), and CIS1 mRNA (*e*) were estimated as described in the legend of **Fig 1**.

SOCS1/JAB, CIS1, and SOCS3/CIS3 were inducible in primary keratinocytes (**Fig 9**). Interestingly, the SOCS family has different functions in keratinocytes and other cell types. In myeloid leukemia M1 cells, IL-6 induces SOCS1/JAB, and SOCS1/JAB inhibits IL-6-induced STAT3 activation (Endo *et al*, 1997; Naka *et al*, 1997; Starr *et al*, 1997). IL-6 strongly enhanced the expression of SOCS3/CIS3 mRNA and AxCACIS3 completely inhibited IL-6-induced STAT3 phosphorylation in keratinocytes (**Figs 2*a* and 6*a,c***); however, SOCS1/JAB mRNA was not induced by IL-6 stimulation and AxCALNLJAB did not affect IL-6-dependent STAT3 phosphorylation (**Figs 2*a* and 6*a,b***). SOCS1/JAB also failed to inhibit IL-6-dependent SOCS3/CIS3 mRNA induction, which was interrupted by the dominant negative STAT3 adenovirus vector (**Fig 8*c,d***). These results suggest that SOCS3/CIS3 is a major negative regulator in the IL-6-induced STAT3 signaling pathway in keratinocytes. These data may also suggest that there is a specific mechanism of IL-6-induced STAT3 activation and a specific role for SOCS3/CIS3 in the IL-6 signaling pathway in primary keratinocytes. Recently, Nicholson *et al* (2000) reported a differential inhibitory mechanism between SOCS1/JAB and SOCS3/CIS3 in the IL-6 receptor, gp130. They reported that SOCS1/JAB binds to JAK1, but does not bind to gp130. In contrast to SOCS1/JAB, SOCS3/CIS3 binds to gp130 directly, but this does not inhibit JAK1 phosphorylation. The fact that SOCS1/JAB does not inhibit the IL-6/STAT3 pathway suggests that STAT3 phosphorylation does not depend on the JAK1 pathway in primary keratinocytes. EGF is reported to activate STAT3 without JAK1 activation (David *et al*, 1996), and IL-6 and EGF activate STAT3 not only via JAK1, but also via src (Lund *et al*, 1999; Zhang *et al*, 2000). In fact, the src inhibitor PP2

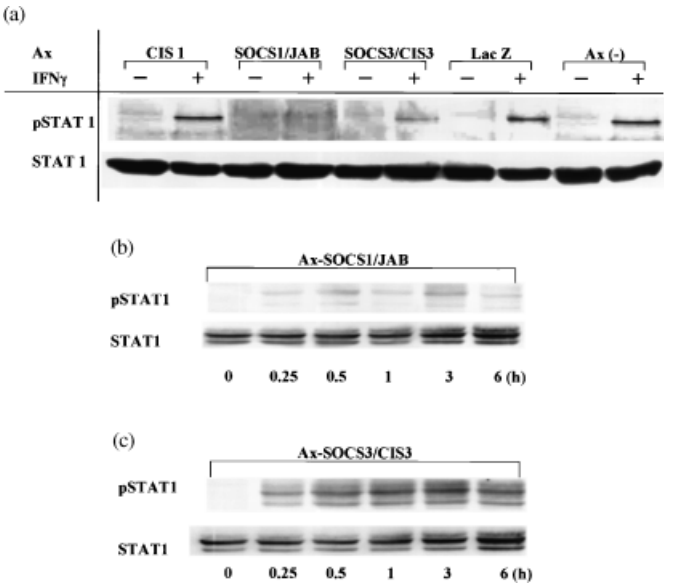

Figure 5. Inhibition of IFN-γ-induced STAT1 activation by SOCS/CIS. Twenty-four hours after transfection with adenovirus vectors, IFN-γ (10 IU per ml) was added to keratinocytes in culture medium; proteins were extracted 15 min after stimulation (*a*) and at the indicated time (*b,c*). Western blot analyses were performed with anti-phosphoSTAT1 and anti-STAT1 antibodies.

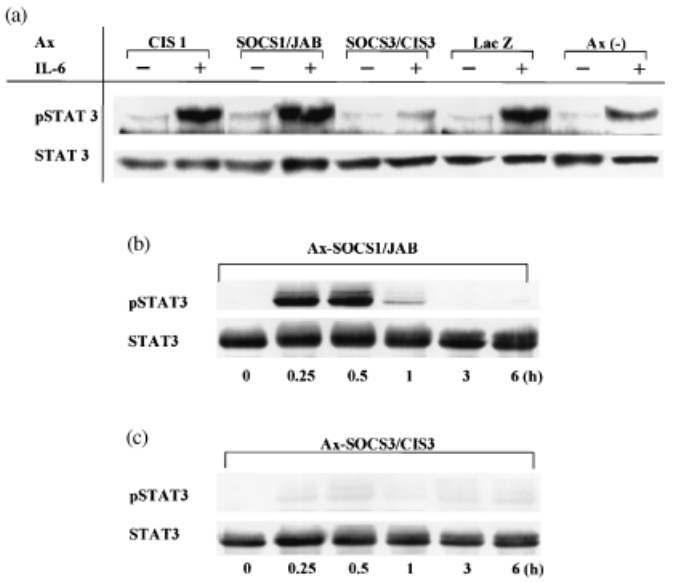

Figure 6. Inhibition of IL-6-induced STAT3 activation by SOCS/CIS. Twenty-four hours after transfection with adenovirus vectors, IL-6 (10 ng per ml) was added to keratinocytes in culture medium; proteins were extracted 15 min after stimulation (*a*) and at the indicated time (*b,c*). Western blot analyses were performed with anti-phosphoSTAT3 and anti-STAT3 antibodies.

inhibited IL-6-dependent STAT3 phosphorylation in primary keratinocytes (data not shown). Hence, the pathway from gp130 to src could be either a main or supplementary pathway for keratinocyte IL-6-dependent STAT3 signaling. This might explain why SOCS1/JAB does not inhibit IL-6-induced STAT3 activation in these cells.

IL-4 induced SOCS1/JAB and CIS1 mRNA, and AxCALNL-JAB inhibited IL-4-dependent STAT6 phosphorylation (**Figs 3*a* and 7*a,b***). These data reveal the effect of SOCS1/JAB in the normal keratinocyte IL-4 signaling pathway. Although a previous report showed that SOCS3/CSI3 inhibits IL-4-dependent STAT6

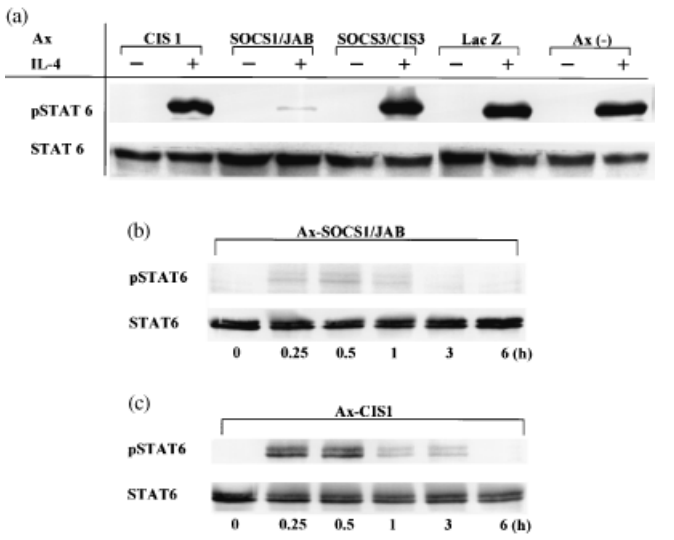

Figure 7. Inhibition of IL-4-induced STAT6 activation by SOCS/CIS. Twenty-four hours after transfection with adenovirus vectors, IL-4 (10 ng per ml) was added to keratinocytes in culture medium; proteins were extracted 15 min after stimulation (*a*) and at the indicated time (*b,c*). Western blot analyses were performed with anti-phosphoSTAT6 and anti-STAT6 antibodies.

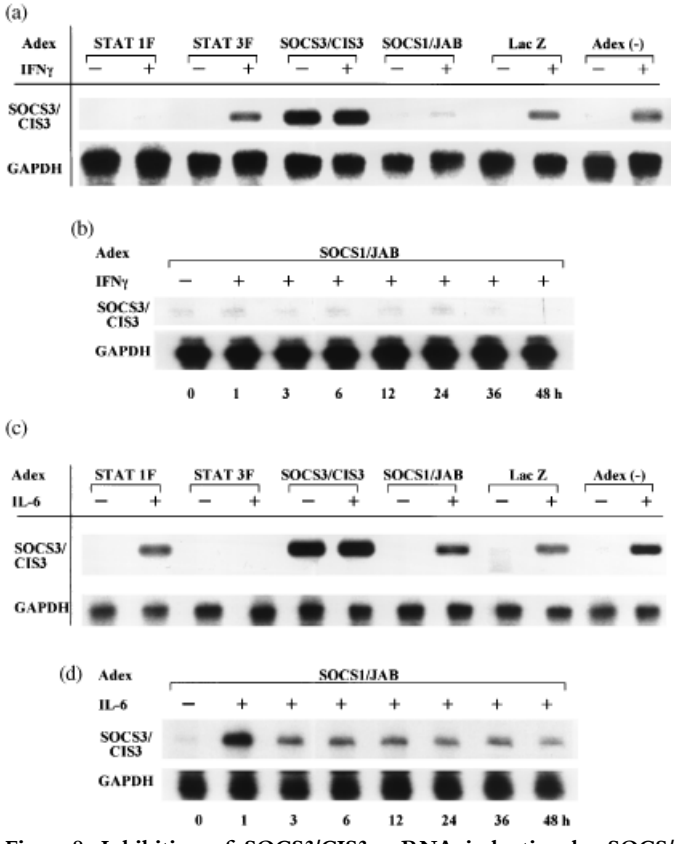

Figure 8. Inhibition of SOCS3/CIS3 mRNA induction by SOCS/CIS. Twenty-four hours after transfection with adenovirus vectors, IFN-γ (10 IU per ml) (*a,b*) or IL-6 (10 ng per ml) (*c,d*) was added to keratinocytes in culture medium; total RNA was extracted 1 h after stimulation (*a,c*) and at the indicated time (*b,d*). The expression of SOCS3/CIS3 mRNA and GAPDH mRNA is indicated.

activation (Haque *et al*, 2000), SOCS3/CIS3 had no effect on IL-4-induced STAT6 phosphorylation in human keratinocytes (**Fig 7*a***). Hence, the SOCS1/JAB protein is a major negative regulator of IL-4-induced STAT6 activation. SOCS1/JAB was

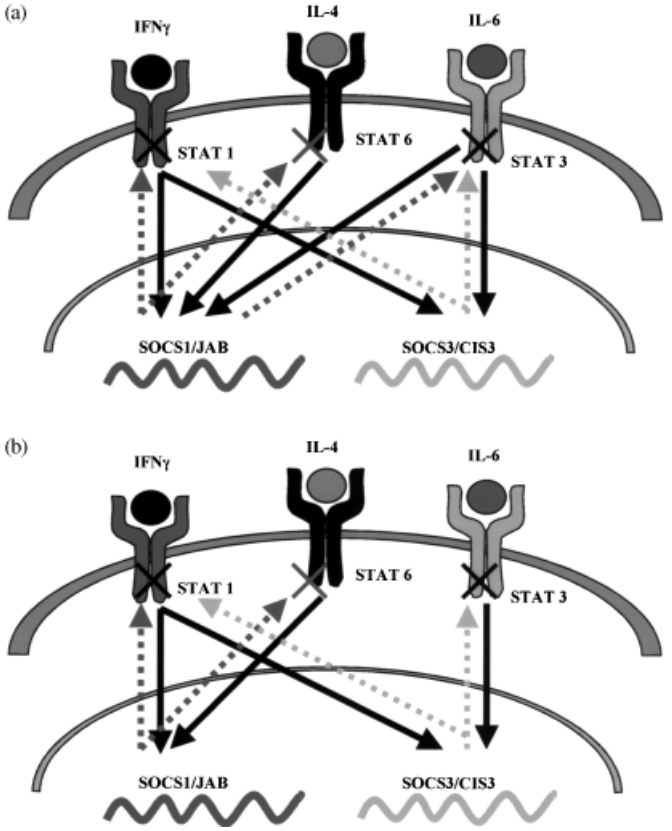

Figure 9. The schema of SOCS/CIS function in monocytes and keratinocytes. This summarizes the induction and function of SOCS1/JAB and SOCS3/CIS3 in monocytes, as previously reported (*a*), and in primary keratinocytes (*b*). SOCS1/JAB is inducible and inhibits the IL-6 to STAT3 signal in monocytes; however, IL-6 did not enhance the expression of SOCS1/JAB in keratinocytes, and SOCS1/JAB did not inhibit IL-6-dependent STAT6 phosphorylation. Solid bars indicate SOCS/CIS induction by cytokines, and broken bars indicate inhibition of STAT activation by SOCS/CIS.

induced by IFN-γ and IL-4 stimulation, and overexpressed SOCS1/JAB inhibited the IFN-γ/STAT1 and IL-4/STAT6 signals (**Fig 9**). Thus, SOCS1/JAB acts as a negative feedback mechanism of IFN-γ and IL-4 stimulation in keratinocytes. IFN-γ is one of the major Th1 cytokines, and IL-4 is a Th2 cytokine. SOCS1/JAB may contribute to homeostasis of the Th1 and Th2 signals in keratinocytes. Huang *et al* (2001) reported that pretreatment with IFN-γ inhibits keratinocyte IL-4-dependent CD60w expression. Combined, our data indicate that SOCS1/JAB regulates the cross-talk between IFN-γ and IL-4. SOCS3/CIS3 was induced by IFN-γ and IL-6 stimulation (Figs 1 and 2), and completely inhibited IL-6/STAT3 signals (**Fig 6*a,c***). This suggests that pretreatment with IFN-γ blocked the IL-6 signal pathway when IL-6 was added after IFN-γ (**Fig 9**); IL-6 appeared to suppress the IFN-γ signal. Thus, the SOCS family may regulate the homeostasis of cytokine signals in primary keratinocytes.

Psoriasis is characterized by hyperproliferation and incomplete terminal differentiation of the epidermis. Previous studies suggested that calcium ions regulate keratinocyte growth and differentiation, and that a calcium gradient exists in the epidermis (Menon *et al*, 1985). Loss of this gradient and altered calcium metabolism have been observed in psoriatic keratinocytes (Menon and Elias, 1991; Karvonen *et al*, 2000). Altered calcium signaling and a persistent inflammatory reaction might be involved in the formation of the characteristic psoriatic epidermis. IFN-γ is secreted from Th1 lymphocyte, and is a possible psoriasis pathogen (Schlaak *et al*, 1994). IFN-γ activates STAT1 and induces the expression of molecules that recruit and activate T cells and other leukocytes in the skin (Barker *et al*, 1990). SOCS1/JAB-deficient mice manifest a thickened epidermis with lymphocyte infiltration into the dermis, much like psoriasis (Metcalf, 1999). In these mice, the IFN-γ signal is not regulated by a negative feedback mechanism involving SOCS1/JAB, and the inflammatory reaction persists. It was reported that administering IFN-γ under normal human epidermis induces psoriasis phenotypes, but these psoriatic reactions resolve quickly (Barker *et al*, 1993; Fransson *et al*, 1996). SOCS1/JAB-deficient mice cannot terminate the IFN-γ-induced inflammatory reaction, which may result in a longitudinal psoriasis phenotype. Th2-derived cytokine IL-4 is abundantly expressed in atopic dermatitis (Del Prete, 1992; Furue, 1994; Ricci, 1994). The balance between Th1 and Th2 cells affects both keratinocyte growth and the expression of chemoattractants by keratinocytes (Fransson *et al*, 1996; Vestergaard *et al*, 2000). SOCS1/JAB acts as a negative feedback mechanism of IFN-γ- and IL-4-induced signal transduction in keratinocytes (**Fig 9**). Thus, SOCS are essential for the regulation of inflammatory reactions, and may act to terminate persistent inflammatory diseases of the skin. Together, the SOCS family is the key regulator of inflammatory reactions, and SOCS family dysfunction is involved in the pathophysiology of many inflammatory skin diseases, such as psoriasis and atopic dermatitis.

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